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# E74-like factor inhibition induces reacquisition of hormone sensitiveness decreasing period circadian protein homolog 1 expression in prostate cancer cells<sup>☆</sup>



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## ABSTRACT

**Purpose:** Initiating as an androgen-dependent adenocarcinoma, prostate cancer (PCa) gradually progresses to a castrate-resistant disease following androgen deprivation therapy with a propensity to metastasize.

**Methods:** In order to resolve the mechanism of castrate-resistant PCa, we performed a cDNA-microarray assay of two PCa cell lines, LNCaP (androgen dependent) and C4-2 (androgen independent). Among them, we focused on a novel Ets transcription factor, E74-like factor 5 (ELF5), the expression level of which was extremely high in C4-2 in comparison with LNCaP both in the microarray analysis and real-time polymerase chain reaction analysis, and investigated the biological role in acquisition of androgen-refractory PCa growth.

**Results:** Western blot analysis and morphological analysis using confocal immunofluorescence microscopy demonstrated that ELF5 was expressed mainly in cytosol both in LNCaP and C4-2. Inhibition of ELF5 expression using ELF5-small interfering RNA in C4-2 induced decreased expression of androgen receptor corepressor, period circadian protein homolog 1, and MTT assay of C4-2 after ELF5 small interfering RNA transfection showed the same cell growth pattern of LNCaP.

**Conclusions:** Our *in vitro* experiments of cell growth and microarray analysis have demonstrated for the first time that decreased expression of period circadian protein homolog 1 due to ELF5 inhibition may induce the possibility of reacquisition of hormone sensitiveness of PCa cells. We suggest that ELF5 could be a novel potential target for the treatment of hormone-refractory PCa patients.

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## 1. Introduction

The Ets family of transcription factors share a highly conserved DNA binding domain, termed the *Ets domain*, first identified in the gag-myb-ets fusion protein of avian leukemia virus E26.<sup>1</sup> Although Ets family members bind to a similar core DNA recognition sequence, GGAA/T, the differences in the sequences flanking the core appear to contribute towards Ets factor binding specificity and,

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hence, function.<sup>2,3</sup> Ets genes encode a family of transcription factors that regulate a spectrum of normal biological activities including development, differentiation, homeostasis, proliferation, and apoptosis. The family consists of 27 members in humans with expression in a variety of tissues; however, most Ets transcription factors are expressed either ubiquitously or restricted to hemopoietic cells. In hemopoietic cells, Ets genes such as the archetypal family member, Ets-1, are crucial for maintaining a normal differentiated cell phenotype. Ets factors integrate extracellular signals at the transcriptional level, resulting in the activation of a large set of target genes.<sup>4</sup>

Prostate-derived Ets factor (PDEF), also known as sterile  $\alpha$  motif-pointed domain containing Ets transcription factor is a relatively novel member of the Ets family of transcription factors. In contrast to normal tissues where the limited tissue-specific expression of

PDEF is generally accepted, its expression in prostate cancer (PCa) is still under investigation. Herein, to investigate the role of Ets family in PCa, we conducted the present study to examine alterations in the PDEF gene expression patterns in C4-2, an androgen-independent PCa cell line, in comparison with LNCaP, an androgen-dependent PCa cell line. We focused on the ELF5 gene, E74-like factor 5, whose expression was much higher in C4-2 than in LNCaP both in the microarray analysis and real-time polymerase chain reaction (PCR) analysis.

The transcriptional regulator E74-like factor 5 (ELF5) has previously been associated with regulation of placentation and alveologenesis, the process by which the mammary gland develops milk-producing acinar structures during pregnancy.<sup>5–7</sup> Unlike most other Ets family members,<sup>8,9</sup> ELF5 is not expressed in hematopoietic compartments, but is restricted to organs such as lung, stomach, kidney, prostate, bladder, and mammary gland.<sup>10</sup> demonstrated that functional evaluation revealed ELF5/ESE-2b expressions contributed to the malignant phenotypes of PCa cells; however, the role of ELF5 in PCa is still unclear.

Our *in vitro* experiments of cell growth and microarray analysis have demonstrated for the first time that decreased expression of period circadian protein homolog 1 (PER1) due to ELF5 inhibition may induce the possibility of reacquisition of hormone sensitivity of PCa cells. Further investigations should be pursued, and we suggest that ELF5 could be a novel potential target for the treatment of hormone-refractory PCa patients.

## 2. Methods

### 2.1. Cell lines

Human PCa cell line LNCaP was purchased from the American Type Culture Collection (Rockville, MD, USA). Human PCa cell line C4-2 was obtained from Dr L.W. Chung (University of Virginia, Charlottesville, VA, USA). Both cells were maintained in RPMI1640 supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Life Technologies, Burlington, ON, Canada) at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>.

### 2.2. RNA isolation and quality measurement

Total RNA of LNCaP or C4-2 was isolated with TRIzol reagent (Life Technologies) in accordance with the manufacturer's instructions. The quality of the RNA was verified with an Agilent 2100 bio-analyzer (Agilent Technologies, Palo Alto, CA, USA).

### 2.3. Microarray analysis and data acquisition

A cDNA microarray containing a set of 17,086 sequence-verified human cDNA clones (Life Technologies) was provided by Genomic Tree, Inc. (Daejeon, Seoul, South Korea). The synthesis of target cDNA and hybridization were performed in accordance with the manufacturer's protocol. Microarray scanning was performed using a GenePix 4000B (Molecular Devices Corporation, Sunnyvale, CA, USA) and analyzed using GenePix Pro6.0 (Molecular Devices). All data normalization and selection were performed using Gene Spring 7.3 (Agilent Technologies).

### 2.4. Real-time polymerase chain reaction analysis

Reverse transcription was carried out with TaqMan RT kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. Briefly, it was used in a volume of 100 µL with 300 ng RNA sample, 15 pmol of oligo deoxythymidine primer and random primer, 10 µL of 10× TaqMan Buffer, 22 µL of 25 mM

MgCl<sub>2</sub>, 20 µL of each 2.5 mM dNTP mix, RNase inhibitor, reverse transcriptase. RT conditions were following: 10 min at 25 °C, 30 min at 45 °C, 5 min at 95 °C. Real-time polymerase chain reaction was performed in an ABI PRISM 7900HT machine (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's instructions in a total volume 25 µL. Cycling conditions were: 2 min at 50 °C, 10 min at 94 °C, 40 cycles of 15 s at 94 °C, 30 s at 58 °C, 1 min at 60 °C. To correlate the threshold (Ct) values from the amplification plots to copy number, a standard curve was generated and nontemplate control was run with every assay. Normalized expression quantity of mRNA was calculated as: Mean of each gene expression quantity/Mean of GAPDH expression quantity × 1000.

### 2.5. Confocal immunofluorescence microscopy

Immunofluorescence examination of ELF5 was performed in LNCaP and C4-2. Both PCa lines were seeded at  $5 \times 10^4$  cells in eight-well chamber slides with a collagen coat (BD Biosciences, San Jose, CA, USA) in RPMI1640 medium supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin. The primary antibody used was anti-ELF5 goat polyclonal antibody (Abcam, Cambridge, UK) and the secondary antibody was Alexa Fluor 488 anti-goat IgG (Molecular Probes, Eugene, OR, USA). Nuclei were stained with PI solution (Dojindo, Kumamoto, Japan). Cells were visualized on an Olympus Fluoview FV500 confocal laser scanning microscope (Olympus, Tokyo, Japan).

### 2.6. Subcellular fractionation and immunoblotting

Purified proteins from four subcellular fractions of LNCaP and C4-2—organelle, cytosolic, cytoskeletal, and nucleic fractions—were obtained using a ProteoExtract Subcellular Proteome Extraction Kit (Merck, Darmstadt, Germany) according to the manufacturer's instructions. Briefly, 5 µg of protein obtained from each subcellular fraction was loaded and separated using 15% sodium dodecyl sulfate-polyacrylamide gel (Bio Craft, Tokyo, Japan) electrophoresis, and then electroblotted onto Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). Immunoblotting was performed with the primary antibodies: anti-ELF5 rabbit polyclonal antibody (Abcam). Immunoblots were exposed to the secondary antibody, anti-rabbit IgG HRP-linked whole antibody, and visualized using ECL Plus chemiluminescence detection reagents (GE Healthcare, Uppsala, Sweden).

### 2.7. Small interfering RNA transfection in C4-2 (real-time PCR)

Human prostate cancer C4-2 cells were grown in RPMI1640 medium supplemented with 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin. Chemically synthesized ELF5 small interfering (si)RNA and negative control siRNA (B-bridge International, Mountain View, CA, USA; [Supplemental Data 1](#)) were diluted in 100 µL RPMI1640 without serum in the wells of a tissue culture six-well plate and mixed gently. Lipofectamine RNAiMAX (4 µL; Invitrogen) was added to each well containing the diluted RNA interference molecules, mixed gently and incubated for 10–20 min at room temperature. The cells (70–90% confluence) were detached by trypsin-EDTA treatment, resuspended in RPMI1640 without antibiotics, and plated into a six-well plate at a density of  $4 \times 10^5$  cells for 1-day transfection,  $2 \times 10^5$  cells for 2-day transfection, and  $1 \times 10^5$  cells for 3-day transfection/3 mL/well containing RNA interference molecules and Lipofectamine mixture. After 24–72 h of incubation, the cells were harvested for real-time RT-PCR analysis of the knockdown level of the exogenous mRNA by siRNA. For

targeting and detection of the endogenous gene level, cells were transfected with the same concentration of negative siRNA control.

## 2.8. Cell growth assay

C4-2, and C4-2 treated with ELF5 siRNA or negative control siRNA were seeded in each well of 96-well plates and maintained in RPMI1640 medium supplemented with 10% FBS, 100 units/mL penicillin and 100 µg/mL streptomycin or RPMI1640 medium supplemented with 10% charcoal/dextran-treated FBS (Thermo Scientific, Waltham, MA, USA), 100 units/mL penicillin and 100 µg/mL streptomycin. Transfections were performed with a mixture of siRNA and Lipofectamine RNAiMAX transfection reagent. The final concentrations of siRNA and Lipofectamine RNAiMAX used in each transfection were 10 nM and 0.12 µL per well, respectively. Cells were cultured for 1 day, 2 days, and 3 days at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>. Each time-course screening assay was performed using triplicate cultures for three independent experiments.

## 2.9. siRNA transfection in C4-2 (microarray analysis)

ELF5 siRNA and negative control siRNA were diluted in 500 µL of serum-free RPMI1640 in one well of a six-well tissue culture plate and mix gently. Lipofectamine RNAiMAX (4 µL) was added to each well containing 30 pmol siRNA and incubated for 20 min at room temperature. The cells (at 70–90% confluence) were detached using trypsin-EDTA, re-suspended in RPMI1640 without antibiotics, and  $2 \times 10^5$  cells were plated in a volume of 3 mL into each well of a six-well plate for 2-day transfection with siRNA and the Lipofectamine RNAiMAX mixture. After 48 h of incubation at 37 °C in a humidified atmosphere containing 5% CO<sub>2</sub>, the cells were harvested for microarray analysis. For targeting and detection of the endogenous gene level, cells were transfected with the same concentration of negative control siRNA.

## 2.10. Statistical analysis

The unpaired Student *t* test was applied to values obtained by *in vitro* experiments. Differences at *P* < 0.05 were considered to be statistically significant.

## 3. Results

### 3.1. cDNA microarray analysis of the hormone-refractory PCa cell line C4-2 and the hormone-sensitive PCa cell line LNCaP

cDNA microarray analysis with a set of 17,086 sequence-verified human cDNA clones was performed on the hormone-refractory PCa cell line C4-2 and the hormone-sensitive PCa cell line LNCaP. In the PCa-related genes, we focused on Ets transcription factors (ELF5, ELF3, EHF, and SPDEF), presented as expression values in Table 1. Among them, there were less than two-fold differences of gene expression levels of ELF3, EHF, and SPDEF between LNCaP and C4-2.

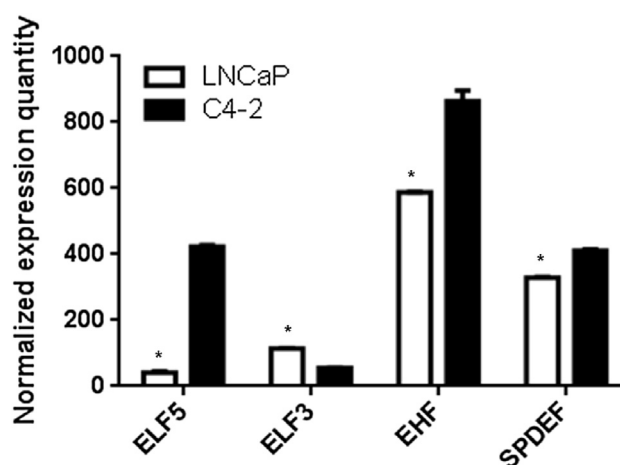


Fig. 1. Real-time polymerase chain reaction analysis of Ets transcription factors (LNCaP vs. C4-2).

By contrast, the ELF5 gene, E74-like factor 5, was more highly expressed in C4-2 than in LNCaP (11,613 vs. 594.96) and the ratio of C4-2 expressing ELF5 relative to LNCaP was >19-fold (19.52).

### 3.2. mRNA quantification of Ets transcription factors using real-time PCR

We next evaluated mRNA levels of ELF5, ELF3, EHF, and SPDEF using real-time PCR analysis. There were less than two-fold differences of mRNA levels of ELF3, EHF, and SPDEF between LNCaP and C4-2, while, consistent with microarray analysis, mRNA of ELF5 in C4-2 was more highly expressed as compared with LNCaP (423.289 vs. 42.272), and the ratio of C4-2 expressing ELF5 mRNA relative to LNCaP was >10-fold (10.01; Fig. 1). Herein, among these Ets transcription factors, we focused on ELF5, whose expression was much higher in C4-2 than in LNCaP, and investigated its biochemical or biological role in the acquisition of androgen-independent growth of C4-2.

### 3.3. Localization of ELF5 expression

Next we examined the localization of ELF5 expression in LNCaP and C4-2 using western blot analysis. We performed western blot analysis on fractions of cytosol, organelles, nucleus, and cytoskeleton separated from each cell line using a ProteoExtract Subcellular Proteome Extraction Kit. ELF5 expression was mainly in cytosol in both LNCaP and C4-2 (Fig. 2A). Then we evaluated the ELF5 expression in cytosol using confocal immunofluorescence microscopy. Consistent with the data obtained by western blot analysis, the microscopy demonstrated ELF5 was expressed mainly in cytosol in both cell lines, however, there was no significant difference in the pattern of ELF5 expression between LNCaP and C4-2 (Fig. 2B).

Table 1  
cDNA microarray analysis in LNCaP and C4-2 cells.

Expression ratio (C4-2/LNCaP)	Gene expression normalized to GAPDH		Gene symbol	Genebank ID	Gene name
	LNCaP	C4-2			
19.52	594.96	11613	ELF5	NM_001422	E74-like factor 5 (ets domain transcription factor)
0.69	2773.91	1925	ELF3	NM_004433	E74-like factor 3 (ets domain transcription factor)
1.39	14813.51	20609	EHF	NM_012153	ESE3 transcription factor
1.56	1152.71	1801	SPDEF	NM_012391	Prostate epithelium-specific Ets transcription factor

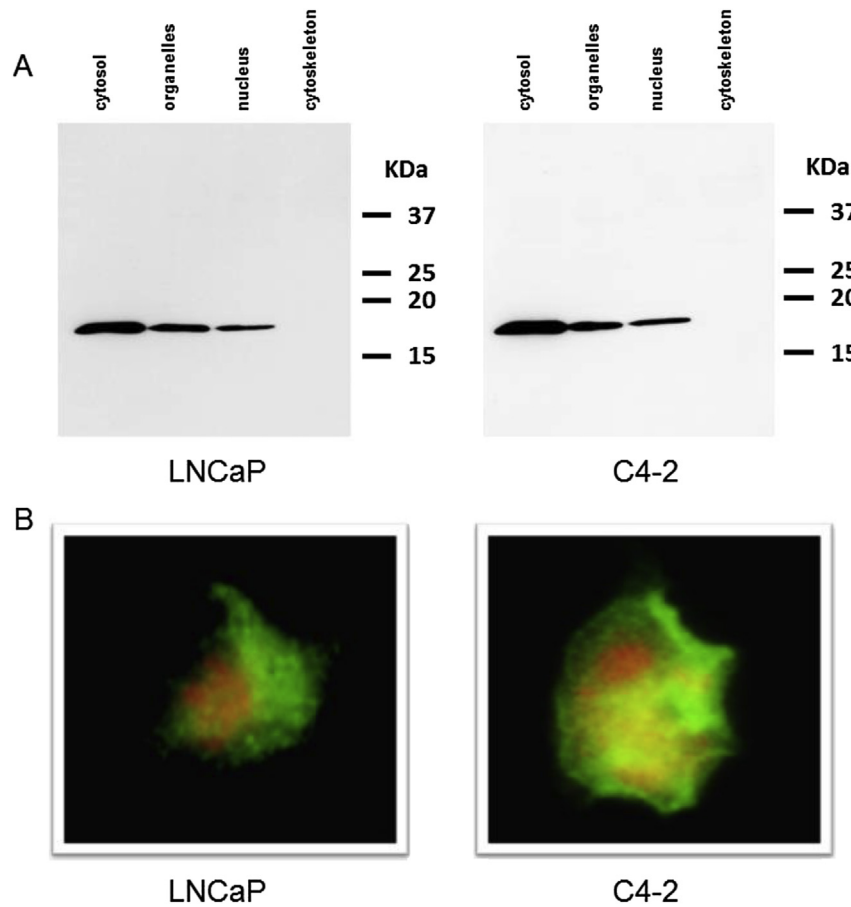


Fig. 2. Representative pictures of (A) western blot analysis and (B) confocal immunofluorescence microscopy in LNCaP and C4-2.

#### 3.4. ELF5 inhibition by siRNA-ELF5 transfection in C4-2 cells

In order to investigate the influence of ELF5 expression on PCA cells, we next transfected chemically synthesized ELF5 siRNA into C4-2 cells. Real-time PCR analysis was used to determine specificity and potency of ELF5 siRNA on inhibiting ELF5 mRNA levels of C4-2 cells as compared with negative control RNA. After the treatment of ELF5 siRNA on C4-2, ELF5 mRNA levels of C4-2 was stably

suppressed at 1 day, 2 days, and 3 days as compared with the treatment of negative control (siRNA/negative control RNA ratio: 1 day 18.2%, 2 days 12.4%, 3 days 13.7%; Fig. 3).

#### 3.5. Acquisition of androgen-dependent cell growth due to ELF5 inhibition in C4-2

We next examined the influence of ELF5 inhibition on cell growth using an MTT assay. In medium containing 10% FBS or 10%

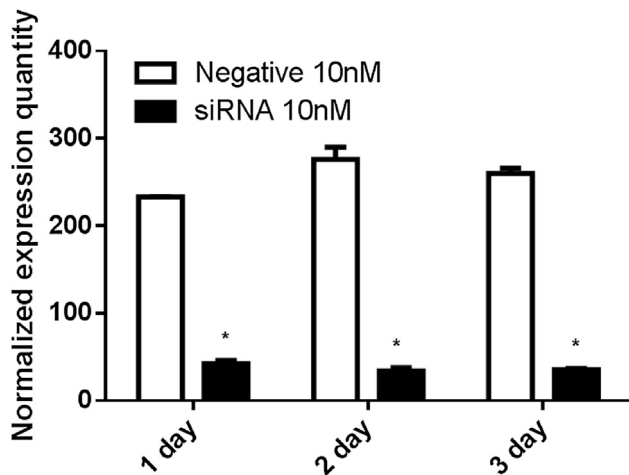


Fig. 3. Real-time polymerase chain reaction analysis after the transfection of chemically synthesized ELF5 siRNA and negative control RNA on C4-2.

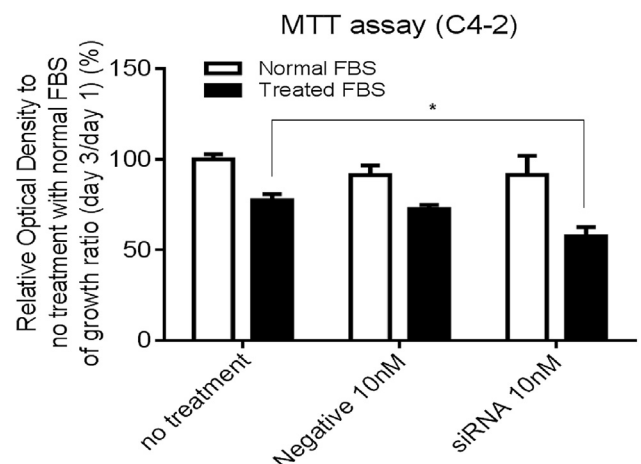


Fig. 4. MTT assay expressed as the growth ratio calculated with values of Day 3/Day 1.

**Table 2**  
cDNA microarray analysis in C4-2 cells.

Expression ratio (Neg 10 nM/siRNA 10 nM)	Gene expression normalized to GAPDH		Gene symbol	Genebank ID	Gene name
	Negative 10 nM	siRNA 10 nM			
0.5	204.3	411.9	PER1	NM_002616	Period homolog 1 (Drosophila)
0.25	374.56	1488.1	ELF5	NM_001422	E74-like factor 5 (ets domain transcription factor)

androgen-depleted serum, the C4-2 growth ratio (Day 3/Day 1) after the treatment of ELF5 siRNA or negative control transfection was compared with those of no treatment. In normal medium, the MTT assay demonstrated that there were no significant differences of C4-2 growth ratio between the treatment of ELF5 siRNA or negative control transfection and that of no treatment. By contrast, in medium with androgen-depleted serum, the C4-2 growth ratio was not affected after the treatment of ELF5 negative control transfection, while C4-2 growth ratio after the treatment of ELF5 siRNA transfection was significantly decreased as compared with that of no treatment (Fig. 4). These results indicated that the treatment of ELF5 inhibition may induce the possibility of re-acquisition of hormone sensitiveness in hormone-refractory PCa cells.

### 3.6. Changes in pattern of gene expression due to ELF5 inhibition by siRNA-ELF5 transfection in C4-2 cells: decreased expression of androgen receptor corepressor, PER1

We next investigated the influence of ELF5 expression on the expression pattern of other genes. We transfected chemically synthesized ELF5 siRNA into C4-2 cells, and investigated the changes in the pattern of gene expression using cDNA array analysis. Among the two-fold-changed genes reported in Supplemental Data 2, we found decreased expression of androgen receptor (AR) corepressor, PER1 in C4-2 after ELF5 inhibition as compared with C4-2 with negative control transfection (Table 2). These results indicate that decreased expression of PER1 due to ELF5 inhibition may induce the possibility of re-acquisition of hormone sensitiveness in hormone-refractory PCa cells, suggesting a promising avenue of treatment for hormone-refractory PCa patients.

## 4. Discussion

Recurrent chromosomal rearrangements are key to development of many hematologic malignancies and sarcomas. PDEF, known as one of relatively novel Ets transcription factors, was originally reported to show strong expression in normal human prostate tissue with weak expression in normal breast and ovary.<sup>11</sup> In PCa, some groups have reported loss of PDEF during tumor progression,<sup>12,13</sup> whereas others showed increased expression of PDEF in PCa in comparison with benign and prostate intraepithelial neoplasia (a premalignant lesion) tissues.<sup>14</sup> PDEF expression is induced via signaling through the AR,<sup>14,15</sup> and it is well-recognized that signaling through AR remains active in castration-resistant advanced PCa.<sup>16</sup>

In the present study, we focused on another novel Ets transcription factor, ELF5, whose expression level was extremely high in C4-2, an androgen-independent PCa cell line, in comparison with LNCaP, an androgen-dependent PCa cell line, both in microarray analysis and real-time PCR analysis. ELF5, also known as ESE2<sup>17</sup> is a member of the epithelium specific subgroup of the large Ets transcription factor family.<sup>18</sup> As mentioned above, ELF5 is found in lung, placenta, kidney, and most prominently in the breast especially during pregnancy and lactation<sup>2,19,20</sup>; however, the mechanism and role in PCa still under investigation. ELF5 was expressed mainly in cytosol in both cell lines, LNCaP and C4-2. There was no significant

difference in the pattern of ELF5 expression between LNCaP and C4-2. In order to resolve the mechanism of ELF5 in hormone-independent environment, we transfected chemically synthesized ELF5 siRNA into C4-2 cells, and investigated cell growth using MTT assay. MTT assay of androgen-independent PCa cell line, C4-2 after ELF5 siRNA transfection demonstrated the same cell growth pattern of androgen-dependent PCa cell line, LNCaP. This cell growth experiment indicated that the treatment of ELF5 inhibition may induce the possibility of re-acquisition of hormone sensitiveness in hormone-refractory PCa cells. We then investigated the influence of ELF5 on the expression patterns of other genes in cDNA microarray analysis after ELF5 inhibition in C4-2 cells. We found decreased expression of androgen receptor corepressor, PER1, in C4-2 after ELF5 inhibition as compared with C4-2 with negative control transfection.

PER1 is a protein that is encoded in humans by the *Per1* gene.<sup>21</sup> Circadian rhythms are driven by a master clock located in the hypothalamic suprachiasmatic nucleus that synchronizes numerous subsidiary oscillators in peripheral tissues. The circadian clockwork in both the suprachiasmatic nucleus and the peripheral cells is regulated by finely tuned transcription–translation feedback loops and post-translational modifications that are maintained by a core set of clock genes.<sup>22–25</sup> The positive feedback loop involves two transcription factors, Clock and Bmal1, which dimerize and bind to E-boxes in the promoters of a large number of target genes. These include their own negative regulators period (PER1, PER2, and PER3) and cryptochrome (Cry1 and Cry2). Circadian rhythms influence many physiologic processes and pathologic conditions including cancer.<sup>26,27</sup> PER1 and PER2 have been reported to be deregulated in several human cancers.<sup>28</sup> In PCa, Cao et al.<sup>29</sup> suggest that activated AR stimulates PER1, which in turn attenuates AR activity, and investigate the connections between clock genes and the AR pathway could benefit the development of new therapeutic strategies for PCa patients.

In our experiments, the expression level of AR in microarray analysis was not affected after ELF5 inhibition in C4-2 cells (Supplemental Data 2: AR <2-fold-changed genes); however, decreased expression of PER1 influenced the mechanism of AR, then AR may occur characteristic conversion from a hormone-independent to hormone-dependent state.

In conclusion, our *in vitro* experiments have demonstrated for the first time that decreased expression of PER1 due to ELF5 inhibition may induce the possibility of re-acquisition of hormone sensitiveness in hormone-refractory PCa cells. Our *in vitro* data regarding re-acquisition of hormone sensitiveness on hormone independent PCa appear to have important implications for future research on PCa.

## Conflicts of interest

The authors disclose no potential conflicts of interest.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.pnrl.2015.02.004>.



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